

# The effect of high doses of folic acid and iron supplementation in early-to-mid pregnancy on prematurity and fetal growth retardation: the mother–child cohort study in Crete, Greece (Rhea study)

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## Abstract

**Purpose** We examined whether high doses of folic acid and iron supplementation in early-to-mid pregnancy affect the risk of preterm birth, low birth weight, and small for gestational age neonates, in the mother–child cohort in Crete, Greece (Rhea study).

**Methods** We included 1,279 women with singleton pregnancies with complete data on supplements use in early-to-mid pregnancy and birth outcomes. Anthropometric measurements at birth were obtained from medical records. Red blood cell folate concentrations in cord blood

were measured in a subsample of the study population ( $n = 58$ ).

**Results** Sixty-six percent of the study participants reported high doses of supplemental folic acid use (5 mg/day), while 21 % reported excessive doses of folic acid use (>5 mg/day) in early-to-mid pregnancy. Daily intake of 5-mg supplemental folic acid was associated with a 31 % decrease in the risk of preterm birth (RR, 0.69; 95 % CI, 0.44, 0.99), 60 % decrease in the risk of delivering a low birth weight neonate (RR, 0.40; 95 % CI, 0.21, 0.76), and 66 % decrease in the risk of delivering a small for gestational age (SGA) neonate (RR, 0.34; 95 % CI, 0.16, 0.73). Daily doses of iron supplementation more than 100 mg were associated with a twofold increased risk for SGA neonates (RR, 2.14; 95 % CI, 0.99, 5.97).

**Conclusion** These findings suggest that high daily doses of supplementary folic acid in early-to-mid pregnancy may be protective for preterm birth, low birth weight, and small for gestational age neonates, while high daily doses of supplementary iron may be harmful for fetal growth.

**Keywords** Folic acid · Iron · Supplements · Pregnancy · Preterm birth · Fetal growth

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## Introduction

During pregnancy, there are increased demands of iron and folate because of placental and fetal growth and development [1]. Pregnant women with a folate deficiency are at an increased risk for various reproductive failures, including neural tube defects [2, 3], while iron deficiency anemia, the late manifestation of chronic iron deficiency, is thought to be the most common nutrient deficiency among pregnant women [4, 5]. A recent Cochrane meta-analysis of 49 trials,

involving 23,200 pregnant women, found that current evidence fails to demonstrate that supplementation with iron alone or in combination with folic acid among women without anemia or with mild/moderate anemia is significantly associated with any substantial beneficial or adverse effects on maternal health, fetal health, or pregnancy outcomes [1]. On the other hand, several studies have drawn attention to the possible side effects of using higher doses of supplemental folic acid and iron in pregnancy than those recommended [6–8].

In developed countries, the use of prenatal folic acid and iron supplements during pregnancy is a common practice, though there are few studies evaluating their effect on birth outcomes, and most of them have focused on mid-late pregnancy [1]. Only few studies assessed the associations peri-conceptionally or in early pregnancy (1st trimester), and findings are not consistent [9–11]. Thus, despite the fact that early pregnancy is the most important period for embryogenesis and fetal programming, relatively little is known about the implications of low, moderate, and high doses of folic acid and iron supplementation during this particular pregnancy period on fetal growth.

We examined whether high daily doses of folic acid and iron supplementation in early-to-mid pregnancy affect the risk of preterm birth, low birth weight, or small for gestational age (SGA) neonates, the mother–child cohort in Crete, Greece (Rhea study).

## Materials and methods

### Study population

The “Rhea” project is a mother–child study that examines prospectively a population-based cohort of pregnant women and their children at the prefecture of Heraklion, Crete, Greece [12]. Female residents (Greek and immigrants) who had become pregnant during the 12-month period starting in February 2007 were contacted at the 4 maternity clinics (2 public and 2 private) in Heraklion and asked to participate in the study. The first contact was made at the time of the first major ultrasound examination (Mean: 11.96 weeks, SD 1.49). The inclusion criteria for study participants were the following: residents in the study area; pregnant women aged >16 years; 1st visit: hospitals or private clinics at the time of the first major ultrasound examination 10–13 weeks of gestation; no communication handicap. The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the ethical committee of the University Hospital in Heraklion, Crete, Greece. Written informed consent was obtained from all women participating in the study.

In total, 1,319 (88 % of the total study population) women were eligible for inclusion in the present analysis, having complete information on supplements intake during early-to-mid pregnancy (14th to 18th week) and, at least, one of the outcome variables (preterm birth, low birth weight, SGA). Only women with singleton pregnancies were finally included in the analysis, resulting in the exclusion of 40 women with multiple births. Hence, a cohort of 1,279 (85.4 % of total study population) women with singleton pregnancies was available for the present analysis.

### Folic acid and iron supplements use in early-to-mid pregnancy

Pregnant women, participating in the “Rhea” study, were asked, using a questionnaire administered by a trained research nurse between 14th and 18th week of gestation (mean: 14.6 weeks; SD 3.2) whether they had taken folic acid and iron supplements, vitamins, or any other dietary supplement since they became pregnant. Supplement users were asked to report the brand name, the dose, and the frequency of intake. The answers were open and not pre-coded so as to ensure that all sources of supplementation were recorded and none was excluded. Reported supplement use (brand, dose, frequency) was converted into daily intake (mg/day) by using dosage information provided on the package of each product. In case that folic acid or iron were obtained from more than one product, the total quantity per micronutrient was estimated by summing up all sources of daily intake, providing the total micronutrient intake from supplements (in mg/day). In Greece, it is not common practice to use multiple micronutrient supplements in pregnancy but iron and folic acid supplements alone, or a combination of the two [11.7 % ( $n = 149$ ) women reported taking iron supplementation products containing small amounts of folic acid]. Several different products were reported for iron supplementation (though all of them contained only iron and not other micronutrients), resulting in a higher variability of the daily iron intake from supplements, while for folic acid, the majority of women ( $n = 1,096$ , 85.7 %) used only one product providing 5 mg of folic acid per dose. Folic acid supplement users were assigned in the following categories: women with no folic acid intake from supplements (0 mg/day), those with daily intake of 5 mg of folic acid from supplements (5 mg/day), and women with daily intake of folic acid from supplements higher than 5 mg, ranging from 6 to 15 mg/day (>5 mg/day). There were no women with daily intake of supplemental folic acid between 0 and 4 mg. Iron supplement users were assigned in the following categories: women with no iron intake from supplements (0 mg/day), daily intake from iron supplements of

up to 100 mg/day (ranging from 5.71 to 100 mg/day), and daily intake from iron supplements higher than 100 mg/day (ranging from 101 to 300 mg/day).

#### Validation of supplemental folic acid use

Red blood cell (RBC) folate concentrations in cord blood were measured by immunoassay with chemiluminescence (Roche kit, Elecsys 2010 analyzer), in an effort to validate the self-reported folic acid supplements' use. This analysis was available for a random subsample of the study population ( $n = 58$ ). The collected cord blood samples were analyzed in batches to reduce between assays variability using internal control (standard) for each batch.

#### Birth outcomes

Information on anthropometric measurements at birth was obtained using the hospital delivery logs and medical records.

#### Gestational age

Gestational age was based on the interval between the last menstrual period and the date of delivery of the baby for 84.2 % of the subjects. When the menstrual estimate of gestational age was inconsistent by 7 or more days with the ultrasound measurement taken in the first trimester of pregnancy ( $n = 231$ , 15.8 %), a quadratic regression formula describing the relation between crown-rump length and gestational age was used instead [13].

#### Preterm birth

Preterm birth was defined at less than 37 weeks among singleton gestations. A spontaneous preterm delivery was defined as a vaginal birth or a birth in which the woman was documented as having been in labor at the time of delivery but the labor was not documented as having been induced and was therefore presumed to be spontaneous. A medically indicated preterm delivery was defined as one that required either an induction of labor or pre-labor cesarean or both [12].

#### Low birth weight

Low birth weight was defined as any newborn with birth weight below 2,500 g.

#### Small for gestational age (SGA)

SGA neonates were defined as live-born infants below the tenth percentile of birth weight for gestational age in a

referent population [14]. In the current analysis, Spanish growth curves were used to calculate SGA neonates as Greek growth curves are not available [15].

#### Potential confounders

Information on potential confounders was obtained using questionnaires completed by personal interview. Potential confounders included characteristics that have an established or potential association with preterm birth, SGA neonates, and use of folic acid and iron supplements in pregnancy including maternal characteristics such as age, education (low level,  $\leq 6$  years of school; medium level,  $\leq 12$  years of school; high level, university of technical college degree), pre-pregnancy weight (kg), pre-pregnancy body mass index (BMI,  $\text{kg}/\text{m}^2$ ), origin (Greek/other), smoking during pregnancy (yes/no), working during pregnancy (yes/no), alcohol intake during pregnancy (g/day), marital status (married/other), parity (primiparous/multiparous), and current pregnancy-related factors such as gestational hypertension or preeclampsia (yes/no), gestational diabetes (yes/no), type of delivery (caesarian/vaginal), infant gender (male/female).

#### Statistical analysis

The primary outcome variables of interest were gestational age, preterm birth, spontaneous preterm birth, medically indicated preterm birth, low birth weight, and SGA. The primary exposure variables were folic acid and iron supplemental intake in early-to-mid pregnancy, using the group of no intake (0 mg/day) as the reference group for each categorical exposure variable. Continuous non-normally distributed variables were displayed as median with interquartile range and were tested using Mann–Whitney nonparametric statistical tests. Normally distributed variables are displayed as mean with standard deviation and tested with t-test, whereas categorical variables were tested with Chi-square test (Chi-square test or Cramer's Chi-square with Monte Carlo correction).

Associations of supplements' use in early-to-mid pregnancy with the outcomes of interest were assessed with univariate analysis and multivariable log-binomial regression models for binary outcomes (i.e., preterm birth, low birth weight, and SGA) or multivariable linear regression models for continuous outcomes (i.e., gestational age) after adjusting for confounders. Potential confounders related with the outcomes of interest in the bivariate models with a  $p$  value  $< 0.2$  were included in the multivariable models. Maternal age, education, smoking during pregnancy and pre-pregnancy BMI were treated as a priori confounders and were included in all multivariable regression models. Risk ratios (RR) with 95 % confidence intervals (95 % CI)

in multivariable log-binomial regression models, and beta-coefficients ( $\beta$ ) with 95 % CI in multivariable linear regression models were computed to estimate the degree of association between the exposure and the outcome of interest.

To account for the possibility of residual confounding, the remaining demographic, lifestyle, and pregnancy characteristics that were available in this data set were then sequentially forced into the parsimonious models to ensure that the estimates associated with supplemental use remained unchanged. Effect modification was evaluated using the likelihood ratio test and stratified models were obtained. All hypothesis testing was conducted assuming a 0.05 significance level and a two-sided alternative hypothesis. All statistical analyses were performed using the PASW computer package (SPSS v18.0 Inc. Chicago, Illinois).

## Results

The majority of women were receiving folic acid ( $n = 1,122$ , 87.7 %) and iron supplements ( $n = 1,090$ , 85.2 %) during early-to-mid pregnancy. A total of 1,023 (80 %) women reported use of both folic acid and iron supplements during pregnancy, 99 (7.7 %) reported single use of folic acid, 67 (5.5 %) single use of iron, and 90 (7.0 %) reported no use of supplements during early-to-mid pregnancy. Dietary intakes of folic acid (median, 0.28 mg/day; IQR, 0.16) and iron (median, 11.3 mg/day; IQR, 6.8) did not differ significantly between supplement users and non-users. Socio-demographic, medical, and lifestyle characteristics by folic acid and iron supplements' use in early-to-mid pregnancy are presented in Table 1. Women who reported prenatal folic acid supplement use were more likely to be highly educated, not working during pregnancy, of Greek origin, and none smokers. Iron supplement users were more likely to be Greek compared with non-users.

In the validation analysis of supplemental folic acid use, women who reported supplemental folic acid use in early-to-mid pregnancy ( $n = 51$ ; 88 %) gave birth to neonates with higher RBC folate concentrations in cord blood (mean, 780 ng/ml; range, 402–1,670 ng/ml), compared with women ( $n = 7$ ; 12 %) who did not use folic acid supplements (mean, 640 ng/ml; range, 358–1,182 ng/ml;  $p$  value = 0.05 from Mann–Whitney test for nonparametric comparisons between groups).

Sixty-six percent ( $n = 849$ ) of the study participants reported daily intake of 5 mg for folic acid from supplements (5 mg/day), while 21.3 % ( $n = 273$ ) reported higher doses of folic acid (>5 mg/day), and 12.3 % ( $n = 157$ ) reported no intake of folic acid in early-to-mid pregnancy.

Similarly, 60.3 % ( $n = 771$ ) of women participating in this study reported iron intake up to 100 mg/day, while 24.9 % ( $n = 319$ ) reported higher daily intake of iron, and 14.8 % ( $n = 189$ ) reported no supplementary iron intake. Women with intakes of 5 mg of folic acid were more likely to take one daily dose of supplemental folic acid, while women with higher intakes reported more than one daily dose. Similarly, women with iron intakes more than 100 mg/day reported taking more than one daily dose compared to those with lower intakes of iron from supplements.

The mean gestational age of our study population was 38 weeks and means of gestational age and birth weight by category of folic acid and iron intake from supplements are presented in Table 2. A total of 146 women (11.4 %) delivered prematurely and of those 105 (8.2 % of the total study population) had spontaneous preterm births. There were 69 (5.4 %) low birth weight neonates and 68 (5.3 %) SGA. High doses of folic acid were related with a lower prevalence of preterm births, low birth weight neonates, and SGA (Table 2).

Daily intake of 5-mg folic acid from supplements was associated with 0.42 weeks increase in gestational age ( $\beta$ , 0.42; 95 % CI, 0.12, 0.71), while for higher daily intakes, the increase was 0.38 weeks ( $\beta$ , 0.38; 95 % CI, 0.03, 0.72), compared with the mean gestational age of women who reported no supplements intake (Table 3). Similarly, intakes of supplemental folic acid equal to 5 mg/day were associated with a decrease in the risk of preterm birth by 31 %, and a decrease by 43 % in the risk of spontaneous preterm birth (RR, 0.69; 95 % CI, 0.44, 0.99; and RR, 0.57; 95 % CI, 0.33, 0.99, respectively), compared with women who did not receive folic acid from supplements. Daily intakes of supplemental folic acid higher than 5 mg were not associated with additional decrease in the risk of preterm birth (Table 3).

Women with intakes of supplemental folic acid equal to 5 mg/day were 71 % less likely to deliver a low birth weight neonate (RR, 0.29; 95 % CI, 0.09, 0.88) and 66 % less likely to give birth to SGA neonates (RR, 0.34; 95 % CI, 0.15, 0.78; Table 4). Daily intakes of supplemental folic acid higher than 5 mg were not associated with a statistically significant decrease in the risk of low birth weight and SGA neonates. To test the possibility of confounding by prematurity, we performed additional analyses excluding all preterm births, and results remained similar to the original analysis (Table 4). Daily supplemental doses of iron higher than 100 mg were associated with a twofold increase in the risk for SGA neonates (RR, 2.14; 95 % CI, 0.99, 5.97).

To test further the effect of iron supplementation on fetal growth, we classified pregnant women into two categories (iron supplement users versus non-users). According to this analysis, iron supplement users had 2.3

**Table 1** Socio-demographic, medical, and lifestyle characteristics, according to folic acid and iron supplement use of women in the mother-child cohort, “Rhea” study in Crete, Greece

	Folic acid supplements use			Iron supplements use		
	Yes ( <i>n</i> = 1,122)	No ( <i>n</i> = 157)	<i>P</i>	Yes ( <i>n</i> = 1,090)	No ( <i>n</i> = 189)	<i>P</i>
Maternal age (years) <sup>a</sup>	29.5 (5.0)	29.1 (5.6)	0.63	29.6 (5.00)	28.8 (5.4)	0.06
Pre-pregnancy BMI (kg/m <sup>2</sup> ) <sup>b</sup>	23.2 (5.2)	23.4 (6.6)	0.78	23.18 (5.03)	23.05 (6.07)	0.96
Dietary folic acid (mg/day) <sup>b</sup>	0.28 (0.15)	0.26 (0.18)	0.10	0.28 (0.15)	0.27 (0.16)	0.16
Dietary iron (mg/day) <sup>b</sup>	11.40 (6.75)	9.92 (6.32)	0.07	11.39 (6.90)	10.77 (6.60)	0.42
Total energy intake (kcal/day) <sup>a</sup>	2,100 (845)	2,060 (945)	0.73	2,094 (854)	2,122 (840)	0.73
Maternal education [ <i>n</i> (%)]						
Low	214 (19.3)	47 (32.0)	<0.001 <sup>c</sup>	214 (19.9)	47 (26.7)	0.11 <sup>c</sup>
Medium	560 (50.6)	74 (50.3)		553 (51.3)	81 (46.0)	
High	332 (30.0)	26 (17.7)		310 (28.8)	48 (27.3)	
Maternal origin [ <i>n</i> (%)]						
Greek	1,027 (92.1)	135 (86.5)	0.02 <sup>c</sup>	998 (92.1)	164 (87.7)	0.04 <sup>c</sup>
Non-Greek	88 (7.9)	21 (13.5)		86 (7.9)	23 (12.3)	
Marital status [ <i>n</i> (%)]						
Married	978 (88.3)	126 (86.3)	0.28 <sup>c</sup>	950 (88.0)	154 (88.5)	0.49 <sup>c</sup>
Other	129 (11.7)	20 (13.7)		129 (12.0)	20 (11.5)	
Parity [ <i>n</i> (%)]						
Primiparous	447 (41.3)	55 (37.4)	0.21 <sup>c</sup>	424 (40.2)	78 (44.3)	0.17 <sup>c</sup>
Multiparous	636 (58.7)	92 (62.6)		630 (59.8)	98 (55.7)	
Working during pregnancy [ <i>n</i> (%)]						
Yes	556 (50.3)	88 (59.9)	0.02 <sup>c</sup>	544 (50.6)	100 (56.8)	0.07 <sup>c</sup>
No	549 (49.7)	59 (40.1)		532 (49.4)	76 (43.2)	
Smoking status during pregnancy [ <i>n</i> (%)]						
Non-smoker	855 (77.3)	103 (70.5)	0.05 <sup>c</sup>	829 (76.9)	129 (74.1)	0.24 <sup>c</sup>
Smoker	251 (22.7)	43 (29.5)		249 (23.1)	45 (25.9)	
Alcohol intake during pregnancy [ <i>n</i> (%)]						
Yes	246 (29.3)	16 (29.6)	0.54 <sup>c</sup>	227 (30.0)	35 (25.5)	0.17 <sup>c</sup>
No	593 (70.7)	38 (70.4)		529 (70.0)	102 (74.5)	
Gestational hypertension and/or preeclampsia [ <i>n</i> (%)]						
Yes	19 (1.9)	4 (3.4)	0.22 <sup>c</sup>	18 (1.9)	5 (3.3)	0.19 <sup>c</sup>
No	980 (98.1)	114 (96.6)		947 (98.1)	147 (96.7)	
Gestational diabetes [ <i>n</i> (%)]						
Yes	92 (9.3)	7 (6.0)	0.16 <sup>c</sup>	86 (9.0)	13 (8.7)	0.52 <sup>c</sup>
No	899 (90.7)	109 (94.0)		871 (91.0)	137 (91.3)	

<sup>a</sup> Normally distributed continuous variables were displayed as mean with standard deviation and were tested using *t*-test

<sup>b</sup> Continuous non-normally distributed variables were displayed as median with interquartile range and were tested using Mann–Whitney nonparametric statistical test

<sup>c</sup> Categorical variables were displayed as *n* (%) and tested with Chi-square test

increased risk of having an SGA neonate (RR, 2.27; 95 % CI, 1.00, 5.14).

We performed an additional analysis for the combined effect of iron and folic acid supplementation in early-to-mid pregnancy on birth outcomes (Table 5). Women who reported no intake of supplements were the reference group. The combination of iron and folic acid supplementation was associated with 0.4 weeks increase in gestational age ( $\beta$ , 0.41; 95 % CI, 0.03, 0.78) and decreased

risk for preterm birth and low birth weight neonates though not statistically significant. There was a contrast in the direction of associations for single iron versus single folic acid supplementation and the risk of SGA, with some suggestion of increased risk of iron though not statistically significant (RR, 2.62; 95 % CI, 0.86, 7.95).

Finally, to test the effect of folic acid and iron intake from diet (food consumption) on birth outcomes, we performed a sensitivity analysis including only women who

**Table 2** Median (IQR) values of gestational age, birth weight and frequency of preterm birth, low birth weight and small for gestational age neonates, according to folic acid and iron intake from supplements in early-to-mid pregnancy, in the mother–child cohort, “Rhea” study in Crete, Greece

	N	Gestational age (in weeks)		Birth weight (in grams)		Preterm birth n (%)	Low birth weight (<2,500 g) n (%)	Small for gestational age n (%)
		Mean	SD	Mean	SD			
Total	1,279	38.2	1.6	3.183	580	146 (11.4)	69 (5.4)	68 (5.3)
Folic acid								
No intake	157	37.8	1.7	3.163	631	26 (16.6)	16 (10.2)	13 (8.3)
5 mg/day	849	38.3	1.5	3.194	585	90 (10.6)	38 (4.5)	42 (4.9)
>5 mg/day	273	38.3	1.6	3.161	560	30 (11.0)	15 (5.5)	13 (4.8)
Iron								
No intake	189	38.0	1.7	3.203	660	28 (14.8)	11 (5.8)	8 (4.2)
≤100 mg/day	771	38.3	1.5	3.174	585	84 (10.9)	37 (4.8)	40 (5.2)
>100 mg/day	319	38.2	1.6	3.196	505	34 (10.7)	21 (6.6)	20 (6.3)

**Table 3** Association between folic acid and iron intake from supplements in early-to-mid pregnancy with gestational age and preterm birth in the mother–child cohort, “Rhea” study in Crete, Greece

	N	Gestational age (weeks)		Preterm birth (n = 146)		Medically indicated preterm birth (n = 41)		Spontaneous preterm birth (n = 105)	
		beta	95 % CI	RR	95 % CI	RR	95 % CI	RR	95 % CI
Folic acid									
No intake	157	Reference <sup>a</sup>		Reference <sup>a</sup>		Reference <sup>a</sup>		Reference <sup>a</sup>	
5 mg/day	849	0.42	0.12, 0.71	0.69	0.44, 0.99	1.88	0.45, 7.88	0.57	0.33, 0.99
>5 mg/day	273	0.38	0.03, 0.72	0.72	0.41, 1.25	0.89	0.17, 4.78	0.71	0.37, 1.34
Iron									
No intake	189	Reference <sup>b</sup>		Reference <sup>b</sup>		Reference <sup>c</sup>		Reference <sup>c</sup>	
≤100 mg/day	771	0.24	−0.04, 0.52	0.83	0.50, 1.39	0.40	0.15, 1.28	0.88	0.47, 1.65
>100 mg/day	319	0.15	−0.17, 0.56	0.76	0.43, 1.36	0.34	0.10, 1.15	0.87	0.44, 1.71

All models are adjusted for maternal age, education, Greek origin, pre-pregnancy BMI (kg/m<sup>2</sup>), smoking status, and parity

beta regression coefficient, CI confidence interval, RR risk ratio

<sup>a</sup> Models are also adjusted for iron intake from supplements

<sup>b</sup> Models are also adjusted for folic acid intake from supplements

<sup>c</sup> Models are also adjusted for child's gender and folic acid intake from supplements

reported no use of folic acid and/or iron supplements during pregnancy. This analysis showed no effect of folic acid and iron intake from diet on fetal growth and preterm birth. Further stratification by dietary intake of folic acid and iron in pregnancy did not modify the effect of supplemental intake of each nutrient on birth outcomes (data not shown).

## Discussion

In this prospective population-based mother child cohort study, we showed that daily intakes of 5 mg supplemental folic acid in early-to-mid pregnancy were associated with lower risk of preterm birth, low birth weight, and SGA

neonates. No additional protection was observed with doses higher than 5 mg/day of folic acid supplementation. High doses of iron supplementation were found to be associated with increased risk for SGA neonates.

Folate is critically important for fetal development as it is required for cell division because of its role in DNA and RNA synthesis [16]. It also acts as a cofactor for many essential cellular reactions including the transfer of single-carbon units, and it is a substrate for a variety of reactions that affect the metabolism of several amino acids, including the transmethylation pathway [17]. A central feature of fetal development is widespread and sustained cell division. As a result of its role in nucleic acid synthesis, the need for folate increases during times of rapid tissue growth [18]. During pregnancy, folate-dependent processes include an increase

**Table 4** Association between folic acid and iron intake from supplements in early-to-mid pregnancy with low birth weight and small for gestational age neonates in the mother–child cohort, “Rhea” study in Crete, Greece

	N	Low birth weight (<2,500 g; n = 69)		Low birth weight: excluding preterm births (n = 27)		Small for gestational age (n = 68)		Small for gestational age: excluding preterm births (n = 60)		
		RR	95 % CI	RR	95 % CI	RR	95 % CI	RR	95 % CI	
<b>Folic acid</b>										
No intake	157	Reference <sup>a</sup>		Reference <sup>a</sup>		Reference <sup>b</sup>		Reference <sup>b</sup>		
5 mg/day	849	0.40	0.21, 0.76	0.29	0.09, 0.88	0.34	0.16, 0.73	0.34	0.15, 0.78	
>5 mg/day	273	0.56	0.26, 1.18	0.32	0.07, 1.16	0.43	0.13, 1.02	0.45	0.15, 1.04	
<b>Iron</b>										
No intake	189	Reference <sup>c</sup>		Reference <sup>c</sup>		Reference <sup>d</sup>		Reference <sup>d</sup>		
≤100 mg/day	771	1.34	0.61, 2.92	2.94	0.73, 10.50	1.76	0.76, 4.96	1.89	0.88, 4.21	
>100 mg/day	319	1.87	0.83, 4.22	3.29	0.80, 12.67	2.14	0.99, 5.97	3.29	0.87, 8.24	

All models are adjusted for maternal age, education, smoking status, pre-pregnancy BMI (kg/m<sup>2</sup>), Greek origin, and parity

CI confidence interval, RR risk ratio

<sup>a</sup> Models are also adjusted for gestational age and iron intake from supplements

<sup>b</sup> Models are also adjusted for iron intake from supplements

<sup>c</sup> Models are also adjusted for gestational age and folic acid intake from supplements

<sup>d</sup> Models also adjusted for folic acid intake from supplements

**Table 5** Association of combined folic acid and iron intake from supplements in early-to-mid pregnancy with gestational age, preterm birth, low birth weight, and small for gestational age neonates in the mother–child cohort, “Rhea” study in Crete, Greece

	N	Gestational age (weeks)		Preterm birth (n = 146)		Low birth weight (<2,500 g; n = 69)		Small for gestational age (n = 68)	
		Adjusted		Adjusted <sup>a</sup>		Adjusted <sup>b</sup>		Adjusted <sup>b</sup>	
		beta	95 % CI	RR	95 % CI	RR	95 % CI	RR	95 % CI
Non-users	90	Reference		Reference		Reference		Reference	
Single folic acid users	99	0.16	−0.32, 0.65	0.62	0.29, 1.33	0.22	0.05, 1.00	0.56	0.13, 2.41
Single iron users	67	−0.08	−0.61, 0.45	0.84	0.40, 1.75	1.27	0.48, 3.35	2.62	0.86, 7.95
Combined folic acid and iron use	1,023	0.41	0.03, 0.78	0.65	0.36, 1.19	0.55	0.26, 1.15	0.92	0.34, 2.48

All models are adjusted for maternal age, education, smoking status, pre-pregnancy BMI (kg/m<sup>2</sup>), Greek origin, and parity

beta regression coefficient, CI confidence interval, RR risk ratio

<sup>a</sup> Also adjusted for child’s gender

<sup>b</sup> Also adjusted for gestational age

in red cell mass, enlargement of the uterus, and growth of the placenta and the fetus. Therefore, additional supply of folic acid during pregnancy is likely to influence fetal growth and gestational age [18].

Up to the present, there are no epidemiological studies that have evaluated the effect of high doses of folic acid supplementation in early-to-mid pregnancy on birth outcomes. Most studies have shown positive effects of daily folic acid intakes of 1 mg or lower on preterm birth and fetal growth restriction [14, 19–21]. Among them, there are few studies that assessed the association between folic acid supplementation in early-to-mid pregnancy and fetal growth or gestational age providing conflicting results [9–11, 22]. Timmermans et al. in the “Generation R” birth

cohort study have shown reduced risk for low birth weight and SGA neonates in women who started supplementation before conception of 0.4–0.5 mg/day, while they found no effect on gestational age [10]. Rolschau et al. have shown lower risk for preterm births and SGA newborns in women receiving folic acid preconceptionally or in the first half of pregnancy in an affluent Northern country [9]. On the other hand, Pastor-Valero et al. in the “INMA” mother–child cohort in Valencia, Spain, found that periconceptional use of folic acid supplements greater than 1 mg/day was associated with decreased birth height and may entail a risk for decreased birth weight [11].

An important finding in the present analysis is that all women had daily intakes of folic acid from supplements

higher than the tolerable upper intake level for adults (1 mg/day), while no additional protection was observed with doses higher than 5 mg/day of folic acid supplementation. This high dose is mainly due to the fact that the majority of women ( $n = 1,096$ , 85.7 %) used only one product providing 5 mg of folic acid per dose. This product is distributed in Greece and Malta and contains 5 mg of folic acid/tablet. Similarly, a previous study in Greece examining the total intake of micronutrients in pregnant women in Greece revealed that the total intake of folic acid was about 3,000  $\mu\text{g}$  [23]. These intakes are extremely high compared to the estimates from other European countries [10, 11]. Although folic acid fortification has been successful in reducing the incidence of neural tube defects [24, 25], the consequences of long-term high folate intake are not known. High doses of folic acid supplementation have been associated with the presence of plasma unmetabolized folate [26], and recent studies have linked high folate intake and increased plasma concentrations to a number of adverse effects [6, 7, 27–30]. Data from a large trial of folate supplementation in pregnancy in UK has shown that in women receiving daily doses of 5 mg folate, all-cause mortality was about a fifth greater, and the risk of deaths attributable to breast cancer was twice as great [6]. With respect to development, an impact of high folate on embryonic development in animals was suggested by two studies in which high folic acid supplementation (20-fold higher than the recommended intake) in animals resulted in embryonic delay and poor protein utilization late in gestation [31, 32]. Because of the potential epigenetic effects of folic acid supplementation on the genome of the offspring [33], further follow-up of this cohort will help to investigate the effects of high folic acid supplementation in pregnancy on child development.

High doses of iron supplementation in early-to-mid pregnancy were found to increase the risk for SGA neonates. Many of the iron preparations available on the Greek market contain 100-mg iron per tablet, and, as a consequence, most of pregnant women were exposed to daily doses of iron of more than 45 mg/day, the upper tolerable limit suggested by the Institute of Medicine [34]. These high doses of antenatal iron supplementation, currently recommended for developing nations (60 to 300 mg of iron/day) and commonly prescribed by obstetricians in industrial societies, may annul the normal haemodilution and even produce abnormally elevated hemoglobin levels in pregnancy [8]. The relationship between high doses of iron supplementation and abnormally high hemoglobin levels merits research because numerous studies have shown that haemoconcentration among pregnant women is associated with an increased risk of their child having a low birth weight [1]. In the present study, iron supplementation was associated with twofold increase in the risk of SGA

neonates. Similarly, a randomized placebo-controlled trial has shown that women receiving high doses of ferrous sulfate [150 g tablet, containing 50 mg of elemental iron] had higher rates of SGA neonates (15.7 %) compared to placebo groups (10.3 %) [8]. Excess iron intake may lead to increased blood viscosity with impaired poor placental perfusion and increased oxidative stress [35], as well as negative influence on the absorption of other minerals [36]. Epidemiological studies have shown that excess iron intake in pregnancy may be associated with increased risk for preeclampsia, gestational diabetes mellitus [7], and infant convulsions [37]. Further follow-up of this cohort will help to investigate the effects of high iron supplementation in pregnancy on child development.

Strengths of the present study include the population-based prospective design, the large numbers of infants with anthropometric measurements at birth, and the high participation rate (90 %). The study population included women from the follow-up of a birth cohort, providing the opportunity to account for the effect of exposures during pregnancy prospectively within the cohort. Moreover, as preterm birth is a heterogeneous rather than a homogeneous entity, we had the possibility to distinguish between spontaneous and medically indicated preterm births. We did not observe any substantial differences between the crude and the adjusted models. Thus, it is unlikely that over-adjustment affected our findings. Participants were unaware of the hypothesis being tested, so misclassification of supplements' intake estimated by the questionnaire is likely to be random with respect to birth outcomes. The exclusion of women who gave birth to twins as well as adjustment for several socio-demographic variables reduced the likelihood of confounding.

There are several limitations in the present study that deserve acknowledgment. We were not able to assess iron deficiency anemia in participating women and could not distinguish between mothers who took supplements pre-conceptionally from those who started once pregnancy was detected. The use of questionnaires to assess micronutrient supplementation may have led to the misclassification of exposure, despite the clear question concerning supplements. However, the validation study we performed in a small sample showed good level of agreement between the estimates from the questionnaire and RBC folate concentrations in cord blood samples. Moreover, we aimed to assess folic acid and iron supplementation in early-to-mid pregnancy to minimize possible recall bias, though we had no information about the proportion of women who began iron or folic acid supplementation after the second trimester of pregnancy. Last, use of supplements in pregnancy is related to socioeconomic status, lifestyle factors (dietary habits) and adverse health behaviors (smoking). Although we incorporated extensive information on



potential social and environmental factors that are associated with fetal growth, there may be other unidentified factors linked with both fetal growth and use of supplements during pregnancy that could explain this association.

In summary, these results suggest that pregnant women in Greece use high doses of folic acid and iron supplementation in early-to-mid pregnancy. These high doses of folic acid from supplements were associated with decreased risk of spontaneous preterm births, low birth weight and SGA neonates, while high doses of iron supplementation may be harmful for fetal growth. Future longitudinal studies are needed to confirm these findings, better understand the complex underlying processes, and examine the effect of high doses of folic acid and iron supplements on child development.

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